Food Microbiology 52 (2015) 59–65

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Comparative inhibitory effects of *Thymus vulgaris* L. essential oil against *Staphylococcus aureus*, *Listeria monocytogenes* and mesophilic starter co-culture in cheese-mimicking models

Rayssa Julliane de Carvalho [a], Geanny Targino de Souza [b], Vanessa Gonçalves Honório [b], Jossana Pereira de Sousa [a], Maria Lúcia da Conceição [a], Marciane Maganani [b], Evandro Leite de Souza [a, *]

[a] Laboratory of Food Microbiology, Department of Nutrition, Health Sciences Center, Federal University of Paraíba, João Pessoa, Brazil
[b] Laboratory of Microbial Process in Microbiology, Department of Food Engineering, Center of Technology, Federal University of Paraíba, João Pessoa, Brazil

ARTICLE INFO

Article history:
Received 5 March 2015
Received in revised form
10 June 2015
Accepted 3 July 2015
Available online 3 July 2015

Keywords:
Thymus
Antimicrobial activity
Pathogenic bacteria
Technological culture
Low-ripened cheese

ABSTRACT

In the present study, we assessed the effects of *Thymus vulgaris* L. essential oil (TVEO) on *Staphylococcus aureus* and *Listeria monocytogenes*, pathogenic bacteria frequently associated with fresh or low-ripened cheeses (e.g., Brazilian *coalho* cheese), and on a starter co-culture comprising *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, which are commonly used for the production of different cheeses. To measure these effects, we determined the minimum inhibitory concentration (MIC) and assessed bacterial cell viability over time in (*coalho*) cheese-based broth and in a semi-solid (*coalho*) cheese model at 10 °C. The MIC for TVEO was 2.5 μL/mL against *S. aureus* and *L. monocytogenes*, while the MIC was 1.25 μL/mL against the starter co-culture. The TVEO (5 and 2.5 μL/mL) sharply reduced the viable counts of all assayed bacteria in cheese broth over 24 h; although, at 5 μL/mL, TVEO more severely affected the viability of the starter co-culture compared with pathogenic bacteria. The addition of 1.25 μL/g of TVEO in the semi-solid cheese model did not reduce the viable counts of all assayed bacteria. At 2.5 μL/g, TVEO slightly decreased the viable counts of *S. aureus*, *L. monocytogenes* and *Lactococcus* spp. in the semi-solid cheese model over 72 h. The final counts of *Lactococcus* spp. in a semi-solid cheese model containing 2.5 μL/mL TVEO were lower than those of pathogenic bacteria under the same conditions. These results suggest that the doses of TVEO used to control pathogenic bacteria in fermented dairy products, especially in low-ripened cheeses, should be cautiously considered for potential negative effects on the growth and survival of starter cultures.

1. Introduction

Essential oils (EOs) are substances naturally synthesized in different plant organs as secondary metabolites characterized as oily aromatic liquids extracted from plant materials (Asbahani et al., 2015). The antimicrobial activity of EOs is promising for the use of these substances on food conservation systems (Bouhdid et al., 2010), such as those applied in dairy products (Asensio et al., 2015; Olmedo et al., 2013). EOs that are considered Generally Recognized as Safe (GRAS) at doses typically used in foods (Burt, 2004; Sousa et al., 2012) and have been approved by the Food and Drug Administration (FDA) for use in foods and drinks include the essential oil from *Thymus vulgaris* L. (TVEO) (FDA, 2009). The efficacy of TVEO in inhibiting a range of pathogenic and/or spoilage food-related bacteria in *in vitro* systems has previously been reported (Ballester-Costa et al., 2013; Kohiyama et al., 2015; Nezhadali et al., 2014). However, studies verifying the inhibitory effects of TVEO against food-related bacteria in food-mimicking systems or even in food matrices are lacking.

Lactococcus species are the major lactic acid bacteria responsible for the acidification of milk during the production of cheeses (López-Díaz et al., 2000). Among the five known *Lactococcus* species, the only bacteria that significantly contribute to the

* Corresponding author. Universidade Federal da Paraíba, Centro de Ciências da Saúde, Departamento de Nutrição, Campus I, 58051-900, Cidade Universitária, João Pessoa, Paraíba, Brazil.
E-mail address: evandroleitesouza@ccs.ufpb.br (E.L. de Souza).

http://dx.doi.org/10.1016/j.fm.2015.07.003
0740-0020/

production of cheeses is *Lactococcus lactis* (Teuber, 1995), and *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* are the most important bacteria for this technological use (Fox et al., 2000). Milk acidification through *Lactococcus* also contributes to the development of improved sensory properties (i.e., taste, flavor and texture) in cheeses (Mills et al., 2010). Moreover, the presence of *Lactococcus* in cheeses has been associated with the increased microbial stability of the products during storage, producing substances (e.g., organic acids, bacteriocins, fat and amino-acid metabolites) for the inhibition of spoilage and pathogenic microorganisms (Coelho et al., 2014).

Cheese is considered a vehicle for foodborne pathogens, in particular, handmade fresh cheeses primarily produced from raw milk. However, this contamination can also occur in cheese produced with pasteurized milk, reflecting post-pasteurization contamination (Brooks et al., 2012; Quero et al., 2014). The microbial contamination of this product is relevant for industry due to economic losses and for public health due to the risk of transmitting potentially pathogenic microorganisms to consumers (Gandhi and Chikindas, 2007). *Listeria monocytogenes* and *Staphylococcus aureus* are involved in many foodborne outbreaks, and these bacteria are considered dangerous threats to the safety of fresh and low-ripened cheeses (Pimentel-Filho et al., 2014). Unripened or low-ripened cheeses infected with *L. monocytogenes* have been associated with major outbreaks of listeriosis worldwide (Coelho et al., 2014). According to reports, 345 foodborne outbreaks in 2011 in Europe were caused by staphylococcal enterotoxins, primarily involving the consumption of cheeses, eggs and mixed foods (Zeleny et al., 2015). Considering the potential of fresh or low-ripened cheeses to harbor pathogenic bacteria, particularly *L. monocytogenes* and *S. aureus*, and the potential impact of these bacteria on food safety, the development of new and effective procedures, such as the inclusion of EOs, for controlling pathogenic bacteria in these products has received much attention from research and industry.

Although the capability of TVEO to inhibit the growth and survival of pathogenic and/or spoilage microorganisms must be deeply considered to develop an innovative food conservation system, the potential inhibitory effects of this compound on beneficial bacterial commonly applied or existing in foods, including those used for technological purposes in fermented products (e.g., mesophilic starter lactic acid bacteria), must also be reconciled. This consideration reflects the fact that the inhibition of technologically beneficial bacteria in foods using an antimicrobial compound could result in unsatisfactory processing or even changes in the desired characteristics of the obtained products. There are only a few reports concerning the effects of spices and/or EOs on starter cultures (e.g., lactic acid bacteria) (Janssen et al., 1987; Kivanç et al., 1991; Shelef, 1983). Moreover, information concerning the effect of TVEO toward starter bacteria commonly used in cheese production remains scarce.

Lactococcus species are used for production of *Coalho* cheese, a semi-hard, medium-to high-moisture (low-ripened) cheese typical of the northeast region of Brazil (Queiroga et al., 2013). This cheese possesses good acceptance among consumers (Garcia et al., 2012) and is produced through simple technology following milk coagulation using rennet or proper coagulating enzymes, which are commonly complemented with selected starter culture mainly composed of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* (Brazil, 1997), and must be consumed in a maximum period of 21 days under cold storage (Garcia et al., 2012; Oliveira et al., 2012). Considering these aspects, in the present study, we assessed the effects of TVEO toward the pathogenic bacteria *S. aureus* and *L. monocytogenes* and a mesophilic starter co-culture comprising *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. These effects were measured (and compared) through the determination of the minimum inhibitory concentration and the assessment of the bacterial cell viability over time in a (*coalho*) cheese-based broth and a semi-solid (*coalho*) cheese model.

2. Materials and methods

2.1. Essential oil

TVEO (batch 178; density at 20 °C, 0.918; refractive index at 20 °C, 1.501, extracted through steam distillation) was purchased from Ferquima Ind. Com. Ltd. (São Paulo, Brazil). The TVEO emulsions were prepared in brain heart infusion broth – BHI (Himedia, India) at a range of concentrations (80–0.312 μL/mL) using Tween 80 (1%, v/v; Sigma–Aldrich, USA) as an emulsifier (Monte et al., 2014). At the highest assayed concentration (1%, v/v), Tween 80 presented no inhibitory effect against the assayed bacterial strains.

2.2. Microorganisms and growth conditions

The *L. monocytogenes* (ATCC 7644) and *S. aureus* (ATCC 6538) strains used in the present study were obtained from the Collection of Reference Microorganisms at the National Institute of Quality Control in Health (FIOCRUZ, Rio de Janeiro, Brazil). The freeze-dried commercial starter co-culture comprising *Lactococcus latis* subsp. *lactis* and *L. lactis* subsp. *cremoris* (R-704, batch 3122631) was obtained from Chr. Hansen Brazil® (Valinhos, Minas Gerais, Brazil). This starter co-culture was chosen as test microorganisms because of its well-known use in the production of low-ripened cheeses (Buriti et al., 2007; Sant'Ana et al., 2013; Garcia et al., 2012; Oliveira et al., 2012; Queiroga et al., 2013). In the present study, the stock cultures were maintained in cryovials at −80 °C.

Inocula of the pathogenic bacteria strains or the starter co-culture used in antimicrobial testing were obtained after preparing suspensions in sterile saline solution (0.85% NaCl p/v), from overnight cultures grown in BHI agar at 37 °C. Each strain or the starter co-culture was grown in BHI broth at 37 °C for 18–20 h (late exponential growth phase), harvested through centrifugation (4500 *g*, 15 min, 4 °C), washed twice in sterile saline solution and re-suspended in BHI broth to obtain standard cell suspensions at which the OD reading at 660 nm (OD_{660}) was 0.1 and 0.8, which provided viable cell counts of approximately 8 log CFU/mL for pathogenic strains and starter co-cultures, respectively, when pour-plated onto BHI agar (McMahon et al., 2008; Wiegand et al., 2008).

2.3. The identification of TVEO constituents

The constituents in TVEO were identified through gas chromatography coupled to mass spectrometry – GC–MS (CGMS-QP2010 Ultra Shimadzu). Analysis through GC–MS was performed under the following conditions: a RTX-5MS capillary column (30 m × 0.25 mm × 0.25 μm); program temperature: 60–240 °C (3 °C/min); injector temperature: 250 °C; detector temperature: 220 °C; carrier gas: helium adjusted to 0.99 mL/min speed; ionizing energy: 70 eV; and mass range (*m*/*z*): 40–500. The spectra bank of GC/MS, NIST/EPA/NIH Mass Spectral Database (Version 1.7) was used to identify the individual essential oil constituents. The quantification of the constituents was obtained after normalizing the areas of each detected constituent, expressed as a percentage area (%).

2.4. Cheese samples and preparation of cheese-based broth

The effects of TVEO on bacterial cell viability were examined using a *coalho* cheese-based broth and a semi-solid *coalho* cheese

model to simulate the environmental conditions of the microorganisms in the studied food matrix. The *coalho* cheese samples (two units of 500 g from the same batch, produced using only enzymatic coagulation with 0.9 mL/L chymosin, without the addition of mesophilic lactic acid culture) used to prepare the cheese-broth and assays using the semi-solid cheese model were purchased from a local retail supermarket in João Pessoa (Brazil). The cheese samples were assessed for hygienic-sanitary conditions according to the current sanitary standards of the Brazilian legislation (Brazil, 2001), which establishes the limits of total and thermotolerant coliforms (45 °C), coagulase-positive *Staphylococcus* (*S. aureus*), *Salmonella* spp. and *L. monocytogenes*. Moreover, the counts of *Lactococcus* spp. in these cheese samples were monitored. The microbiological analyses were performed according to the standard methods described elsewhere (APHA, 2001), and the obtained data showed a satisfactory sanitary quality for the cheeses samples according to the established criteria, highlighting the absence of *L. monocytogenes*, *Salmonella* spp. and *S. aureus*. The viable *Lactococcus* spp. counts in the cheese samples were consistently near 3 log CFU/mL.

To obtain the cheese-based broth, 160 g of *coalho* cheese (previously macerated) was added to 1000 mL of sterile distilled water and hand-mixed using a sterile glass stem for 5 min to ensure even homogenization. Subsequently, the mixture was placed in a thermostatic water bath (42 °C, 50 min) and vacuum-filtered using Whatman no. 1 filter paper. The obtained filtrate was sterilized through autoclaving (121 °C, 1.1 atm, for 15 min) (Neviani et al., 2009). The filtered broth was stored in 50-mL aliquots at −20 °C, and when required, an aliquot was thawed under refrigeration (7 ± 1 °C) and used for subsequent assays.

The physico-chemical characterization (moisture, ashes, fats, proteins, and carbohydrates) of the cheese-based broth and *coalho* cheese used as a substrate in antimicrobial assays was performed according to standard procedures described elsewhere (AOAC, 2012). The values of the assessed parameters were moisture 98.4 g/100 g; ashes 0.37 g/100 g; fats 0.39 g/100 g; proteins 0.7 g/100 g; and carbohydrates 0.23 g/100 g for cheese-based broth; and moisture 46.5 g/100 g; ashes 4.57 g/100 g; fats 6.35 g/100 g; proteins 22.17 g/100 g; and carbohydrates 20.40 g/100 g for cheese.

2.5. Determination of the minimum inhibitory concentration (MIC) of TVEO

A microtiter plate assay was used to determine the MIC of TVEO according to the standard method (CLSI, 2006), with minor modifications related with the use of a stain to detect bacterial growth/survival. Approximately 50 μL of each of the tested TVEO emulsion (80−0.312 μL/mL) was dispensed into each well of a 96-well microplate containing 100 μL of BHI broth. Subsequently, 50 μL of bacterial suspension (6 log CFU/mL) were added to each well. The microplate was loosely wrapped with cling wrap to prevent bacterial dehydration and TVEO volatilization. Each plate included controls without TVEO. The system was incubated at 37 °C for 24 h. Subsequently, a 30-μL aliquot of resazurin (0.01%, m/v) (Inlab, Brazil) prepared in aqueous solution (Aguiar et al., 2015), was added to each well. The color changes were visually assessed after 20 min at 37 °C. Bacterial growth was indicated as color changes from purple to pink (or colorless). The MIC values were confirmed as the lowest concentrations capable of inhibiting bacterial growth.

2.6. Effects of TVEO on bacterial cell viability in cheese-based broth

The effects of different TVEO concentrations (1.25, 2.5 and 5 μL/mL) on the cell viability of each tested bacterial suspension (each pathogenic bacterium or starter co-culture) when inoculated in *coalho* cheese-broth were assessed over 24 h using the viable cell count method. Initially, 150 μL of the tested bacterial suspension (approximately 8 log CFU/mL) was inoculated into 2850 μL of separated cheese-broth samples containing TVEO at the desired final concentrations. The different systems (final viable cell counts of approximately 6 log CFU/mL) were gently hand-shaken for 30 s, and subsequently incubated at 10 °C (proper storage temperature of *coalho* cheese) for 24 h. At intervals of 0 (just after homogenization), 1, 2, 4, 8, 12 and 24 h of post-incubation (exposure time), an aliquot of 100 μL of each system was serially diluted in sterile saline solution, and subsequently 20 μL of each dilution was added to M17 agar base (Himedia, India) to count viable *Lactococcus* spp., Baird Parker agar (Himedia, India) supplemented with 50 mL/L of egg yolk emulsion containing potassium tellurite (3.5%) (Himedia, India) to count *S. aureus* and *Listeria* Agar Base containing selective supplement for *Listeria* II (Himedia, India) to count *L. monocytogenes*, using a microdrop inoculation technique (Herigstad et al., 2011). Control systems without the addition of TVEO were similarly assayed. The plates were incubated at 37 °C for 24−48 h. Plates inoculated with aliquots collected from broth containing TVEO were incubated for an additional 24 h at an adequate temperature compared with samples collected from control assays. The results are expressed as log CFU/mL.

2.7. Effects of TVEO on bacterial cell viability in semi-solid cheese-model

The effects of different TVEO concentrations (2.5 and 5 μL/mL) on the cell viability of each tested bacterial suspension (each pathogenic bacterium or starter co-culture) inoculated in *coalho* cheese slurry samples were assessed over 72 h using the viable cell count procedure. In each of four different systems (each referring to a specific cold storage period, as follow 0 h: 1st system - just after homogenization of the system, 24 h: 2nd system, 48 h: 3rd system and 72 h: 4th system) containing 20 g of macerated *coalho* cheese (taken from the same macerate cheese pool), 10 mL of sterile saline solution, 1 mL of the tested bacterial suspension (approximately 8 log CFU/mL) and the desired final concentration of TVOE were added. The systems were mixed using a sterile glass stem for 5 min to ensure even homogenization (final viable cell counts of approximately 6 log CFU/mL) and incubated at 10 °C. At each pre-established storage period, 70 mL of sterile saline solution were added to the corresponding system, homogenized and serially diluted (10-1−10-5) in sterile saline solution. Subsequently, 20-μL aliquots of each dilution were dispensed onto M17 agar base (Himedia, India) to count viable *Lactococcus* spp., Baird Parker agar (Himedia, India) supplemented with 50 mL/L of the egg yolk emulsion containing potassium tellurite (3.5%) (Himedia, India) to count viable *S. aureus* and *Listeria* Agar Base containing selective supplement for *Listeria* II (Himedia, India) to count viable *L. monocytogenes*, using the microdrop inoculation technique (Herigstad et al., 2011). Control systems without the addition of TVEO were similarly assayed. The plates were incubated at 37 °C for 24−48 h. Plates inoculated with aliquots collected from cheese-models containing TVEO were incubated an additional 24 h longer at adequate temperature compared with samples collected from control assays. The results are expressed as log CFU/g.

2.8. Statistical analysis

All assays were performed in three independent experiments in triplicate, and the results are expressed as an average of the assays. For MIC determination assays, the results are expressed as modal values because the MIC values were the same in all repetitions (McMahon et al., 2008). For the viable cell counts assays, statistical

analysis was performed to determine significant differences ($p \leq 0.05$) – in viable cell counts of test organisms when exposed to the different concentrations of TVEO over time in each grow media tested – using ANOVA, followed by post-hoc Tukey's test. Sigma Stat 3.5 computer software (Jandel Scientific Software, San Jose, California) was used for the statistical analysis of the data.

3. Results and discussion

The GC–MS analysis of TVEO identified 24 different constituents (Table 1). The constituents detected at the highest amounts in TVEO were thymol (43.19%), *p*-cymene (28.55%), γ-terpinene (6.36%), linalool (5.57%) and carvacrol (3.14%). Other constituents, such as α-pinene (2.60%), myrcene (1.4%), eucalyptol (1.29%), camphor (1.72%) and caryophyllene oxide (1.43%), were detected in minor amounts. A variety of other constituents were detected in amounts lower than 1%. These findings are similar to the results of Al.Maqtari et al. (2011), who examined the same essential oil. However, previous studies have also demonstrated great variation in the amounts of compounds often detected as major constituents in samples of TVEO, such as 17.4–71% thymol; 10–56% *p*-cymene, 2.6–85.5% limonene and 12.1–24.5% carvacrol (Burt, 2004; Omidbeygi et al., 2007; Razzaghi-Abyaneh et al., 2008; Sacchetti et al., 2005). The variability in composition among different EO samples obtained from the same vegetal species might reflect differences in the raw materials (dried or fresh) used for extraction, variable ecological and geographical conditions, the age of the plant, harvesting time and methods used for extraction (Kohiyama et al., 2015). These variations in the chemical profile of OEs might influence the antibacterial activity spectrum and intensity (e.g., MIC values and decrease in bacterial cell population) of these substances.

The MIC for TVEO against both *S. aureus* and *L. monocytogenes* was 2.5 μL/mL, while the MIC against the mesophilic starter co-culture comprising *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* was 1.25 μL/mL. Regarding the observed MIC values, the assayed mesophilic starter culture presented higher sensitivity to TVEO compared with both *L. monocytogenes* and *S. aureus*. The MIC of TVEO toward the starter co-culture was one-fold lower than the MIC toward the assayed pathogenic bacteria. Initially, the higher sensitivity of the bacteria comprising the tested starter co-culture was cautiously considered because there is a well-accepted intrinsic two-fold variability in the results achieved for MIC assays, determined through dilution in media (Smith et al., 2007). The MICs for TVEO against *L. monocytogenes* and *S. aureus* obtained in the present study were similar to the findings of previous studies of the same essential oil and target bacteria (Al.Maqtari et al., 2011; Nezhadali et al., 2014; Tohidpour et al., 2010). However, the MICs for TVEO against the mesophilic starter lactic acid co-culture tested were distinct of those observed in previous studies, when lower and higher MIC for TVEO were observed against single cultures of *L. sakei* (0.125–0.5 μL/mL) (Gutierrez et al., 2009), *Lactobacillus plantarum*, *L. delbrueckii* and *L. lactis* (>10 μL/mL) (Di Pasqua et al., 2005).

Some authors have associated the strong antimicrobial activity of TVEO (regarding the low MICs often detected) with the particular profile of major constituents in this substance (Carović-Stanko et al., 2010). The activity rank of the EOs individual constituents possessing the highest antimicrobial properties is phenols > aldehydes > ketones > alcohols > ethers > hydrocarbons (Ballester-Costa et al., 2013). This classification supports the low MICs, i.e., effective antibacterial activity (Van Vuuren, 2008), observed for the TVEO examined in the present study because thymol (monoterpene possessing a phenolic ring) was a major constituent. The primary site of the toxic action of EOs in bacterial cells is the cytoplasmic membrane, and this action has been associated with the hydrophobicity of the individual constituents of EOs, facilitating the partitioning of these constituents into the bacterial lipid bilayer, thereby disturbing the bacterial structure and increasing permeability to protons, ions and other cell constituents. Particularly, thymol (major constituent of TVEO) damages the bacterial cytoplasmic membrane, resulting in the collapse of the proton motive force, depletion of the ATP pool and eventual cell death (Ballester-Costa et al., 2013; Nowak et al., 2012).

Considering that most of the available studies have considered the inhibitory effects of EOs toward pathogenic microorganisms to determine potential doses to use as antimicrobials in foods, the MIC value of TVEO against the assayed pathogenic bacteria was selected as a major parameter for the selection of the different TVEO concentrations used in assays of bacterial survival (cell viability) in cheese-based broth over time (at 10 °C). Thus, the assays to observe the effects of TVEO on bacterial cell viability were performed using cheese-based broth containing 1.25 (1/2 × MIC), 2.5 (MIC) or 5 μL/mL (2 × MIC) TVOE (Fig. 1A–C). Observing the microbial behavior in food-based broth might be useful for studies on food matrices because these liquid models might facilitate the optimization of the final application of EOs and closely reflect the conditions in food products.

The effect of 1.25 μL/mL TVEO on the cell viability of *L. monocytogenes* and starter co-culture in cheese-based broth was similar, with reductions of approximately 1 log CFU/mL in initial counts (zero time) after 24 h of exposure (Fig. 1B and C). This TVEO concentration showed no decrease in the viable cell counts (counts) of *S. aureus* in cheese-based broth after 24 h of exposure, as the counts were similar ($p > 0.05$) over the assessed time intervals (Fig. 1A). When TVEO was added to the cheese-based broth at 2.5 μL/mL, a decrease in the counts of 0.9 log CFU/mL and 1.3 log CFU/mL for *S. aureus* and *L. monocytogenes*, respectively, was observed after 24 h of exposure. However, at 2.5 μL/mL, TVEO sharply decreased the counts of the assayed starter co-culture after 4 h of exposure, and counts near 2 log CFU/mL were observed after 24 h of exposure (a reduction of approximately 4 log cycles in the initial counts). When TVEO was added to the growth medium at 5 μL/mL, the counts of *S. aureus* and *L. monocytogenes* presented a sharp decrease over time, with kill-time curves similar to those

Table 1
CG–MS analysis of the essential oil from *Thymus vulgaris* L.

Peaks	Compound	Area (%)
1	Tricyclene	0.15
2	α-Thujene	0.05
3	α-Pinene	2.60
4	Camphene	0.84
5	Myrcene	1.40
6	Pseudolimonene	0.06
7	α-Terpinene	0.06
8	*p*-Menth-1-ene	0.05
9	*p*-Cymene	28.55
10	Limonene	0.14
11	Eucalyptol	1.29
12	Linalol	5.57
13	cis-Linalol oxide	0.17
14	trans-Linalol oxide	0.20
15	γ-Terpinene	6.36
16	Camphor	1.72
17	Isoborneol	0.20
18	Borneol	1.36
19	Terpinene-4-ol	0.96
20	*p*-Menthane-1,2,4-triol	0.15
21	Thymol	43.19
22	Carvacrol	3.14
23	Caryophyllene	0.35
24	Caryophyllene oxide	1.43

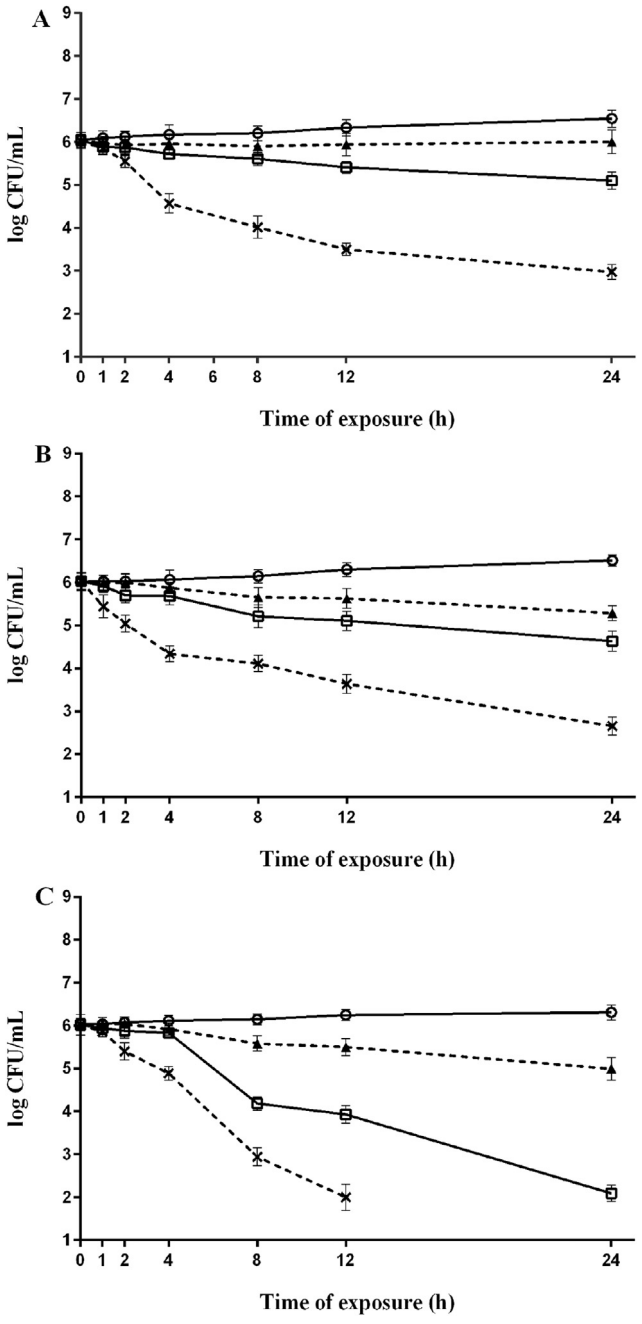

Fig. 1. Viable cell counts of*S. aureus* (A), *L. monocytogenes* (B) and mesophilic starter co-culture (C) in*coalho* cheese based-broth at 10 °C as a function of *Thymus vulgaris* L. essential oil concentration: (○): 0 μL/mL; (▲): 1.25 μL/mL; (□): 2.5 μL/mL; (×): 5.0 μL/mL. Detection limit of the test: 2.0 log CFU/mL.

obtained for the mesophilic co-culture when exposed to TVEO at 2.5 μL/mL. At 5 μL/mL, TVEO decreased the counts of the starter co-culture to 2 log CFU/mL after 12 h of exposure, although a 3 log cycle reduction of the initial population (>99.9%) was detected after 8 h of exposure. Systems containing TVEO consistently presented lower viable cell counts ($p \leq 0.05$) over time compared with control assays, except for the *S. aureus* counts in broth containing TVEO at 1.25 μL/mL. Bacterial cells cultivated in control systems presented a slight increase in the counts over the assessed time interval (0.3–0.7 log increase after 24 h compared with initial counts – time zero). These results suggested that at 2.5 μL/mL, TVEO established a bactericidal effect [≥3 log CFU/mL reduction of initial count, i.e., ≥99.9% reduction] (Laplante, 2007) against the starter co-culture after 24 h of exposure, and this effect was only observed toward both *S. aureus* and *L. monocytogenes* when TVEO was tested at 5 μL/mL (after 24 h). Moreover, at 5 μL/mL, TVEO showed a bactericidal effect against the starter co-culture after 12 h of exposure. Overall, the assayed co-culture presented greater sensitivity than *S. aureus* and *L. monocytogenes* when cultivated in cheese-based broth.

Considering that the incorporation of TVEO at 5 μL/mL in the cheese-based broth severely affected the viability of the bacteria

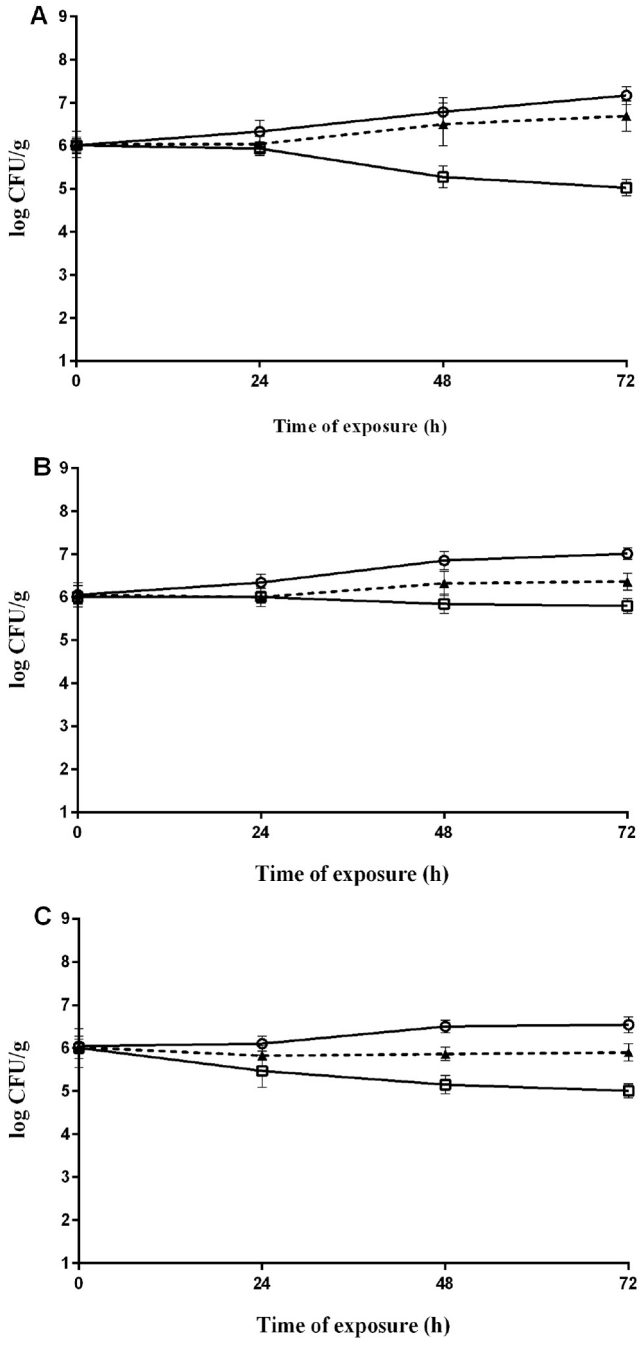

Fig. 2. Viable cell counts of *S. aureus*(A), *L. monocytogenes* (B) and *Lactococcus* spp. (C) in semi-solid *coalho* cheese model at 10 °C as a function of *Thymus vulgaris* L. essential oil concentration: (○): 0 μL/mL;(▲): 1.25 μL/mL; (□): 2.5 μL/mL. Detection limit of the test: 2.0 log CFU/mL.

comprising the tested mesophilic starter co-culture in a shorter exposure time compared with *S. aureus* and *L. monocytogenes*, assays of bacterial survival in a semi-solid *coalho* cheese model (at 10 °C) were performed over 72 h using TVEO at 1.25 and 2.5 μL/g (Fig. 2A–C). In contrast with the results of bacterial behavior observed in cheese-broth, the addition of TVEO at 1.25 μL/g in the semi-solid cheese model did not reduce the counts of *L. monocytogenes*; however, a slight increase in counts was observed from 24 h exposure onward (Fig. 2B). Similar results were obtained for *S. aureus* over time (Fig. 2A). Under similar conditions, the counts of *Lactococcus* spp. were consistently close to the initial bacterial population (approximately 6 log CFU/mL) added to the growth matrix (Fig. 2C). The incorporation of TVEO at 2.5 μL/g in the semi-solid cheese model decreased the counts of *S. aureus*, *L. monocytogenes* and *Lactococcus* spp. after 72 h of exposure, which varied from 0.3 to 1.0 log CFU/g with respect to the initial counts. The final counts of *Lactococcus* spp. in systems containing TVEO at 2.5 μL/mL were lower ($p \leq 0.05$) than the counts observed for *L. monocytogenes*. The results of bacterial behavior in cheese-broth and in the semi-solid cheese model are consistent with the results of the MIC determination assays, suggesting that at lower concentrations, TVEO greater affect *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* compared with *S. aureus* and *L. monocytogenes*. Consistent with results found in cheese-broth, bacteria strains cultivated in semi-solid cheese control systems presented a linear and slight increase in counts over the assessed time period.

Overall, the inhibitory effects of TVEO against the tested target bacteria were higher in cheese-based broth than in the semi-solid cheese model. This difference could be associated with the characteristics of each experimental medium used in these assays, because the composition of the food-mimicking system could impact the antimicrobial efficacy of EOs. Systems containing higher amounts of protein and fat, such as the semi-solid cheese-model, decrease the efficacy of EOs (Gutierrez et al., 2008). Proteins can form complexes with phenolic constituents present in EOs, while fatty acids enclose the hydrophobic individual constituents of EOs, making these components unavailable to attack target cells (Burt, 2004). The physical structure of the semi-solid cheese model could also impair the even dispersion of TVEO in the system, making it difficult to make contact with microbial cells in some specific areas (Gutierrez et al., 2008, 2009). Moreover, the higher availability of nutrients in the semi-solid cheese model compared with cheese-based broth might facilitate the rapid repair of damaged bacterial cells (Burt, 2004; Nazer et al., 2005).

Although researchers have suggested that as starter cultures, lactic acid bacteria are relatively resistant to the toxic effects of spices or even some EOs (Kivanç et al., 1991), showing stimulatory effects on these organisms and enhanced acid production (Shelef et al., 1980; Tiwari and Pandey, 1981), in the present study, TVEO decreased the viability of *Lactococcus* cells. However, the stimulatory effects on starter cultures have been associated with natural spices rather than with their EOs, extracts or individual constituents (Kivanç et al., 1991). A previous study showed that the EOs from cumin (300 and 600 ppm) and oregano (150, 300 and 600 ppm) inhibited the growth of *L. plantarum* and *Leuconostoc mesenteroides* in synthetic medium for 5 days and inhibited acid production by these bacteria (Kivanç et al., 1991). Other studies assessing the effects of the addition of the EOs from oregano or rosemary on the parameters associated with the fermentation of fresh cheeses during storage have shown that cheeses containing EOs presented higher pH values, lower production of organic acids and low amounts of indicators of chemical oxidation compared with cheeses without EOs (Asensio et al., 2015; Olmedo et al., 2013) and low total mesophilic bacteria counts (Olmedo et al., 2013). The authors proposed that the addition of the EOs to cheeses presents a protective effect against deterioration during storage, although the potential effects on beneficial lactic acid bacteria typically observed in the studied matrices have not been assessed. Overall, we should consider that the application of TVEO in fermented dairy products (such as, cheeses) in doses enough to control pathogenic bacteria could negatively affect the growth and survival of starter cultures comprising lactic acid bacteria and speculate a potential decrease in acid production, affecting the proper sensory characteristics and safety of the products.

4. Conclusion

TVEO presented inhibitory activity against pathogenic bacteria *S. aureus* and *L. monocytogenes*, which are often associated with fresh and low-ripened cheese, and a co-culture comprising *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* used in the manufacture of this product. However, the growth of the starter co-culture was more severely affected by TVEO, considering the MIC values and the behavior of test bacteria when challenged with this essential oil at different concentrations in a *coalho* cheese-based broth and in semi-solid *coalho* cheese model. Thus, the doses of TVEO proposed to control pathogenic bacteria in fermented dairy products, particularly in low-ripened cheeses, could be cautiously established because of the potential negative effects on the growth and survival of beneficial lactic acid starter cultures used in the production of these foods.

Acknowledgments

The authors would like to thank the CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil) for a scholarship awarded to the authors R.J. de Carvalho and G.T. de Souza and the Unity for Characterization and Analysis (Institute of Research in Drugs and Medicines, Federal University of Paraíba, João Pessoa, Brazil) for analysis of essential oils individual constituents.

References

Aguiar, U.N., Gonçalo de Lima, S.G.L., Rocha, M.S.R., Citó, A.M.G.L., Sousa, A.J.P., Silva, R.M., Silva, I.S.A., Costa, J.G.M., 2015. Chemical composition and modulation of antibiotic activity of essential oil of *Lantana caatingensis* M. (Verbenaceae). Ind. Crop. Prod. 74, 165–170.

Al.Maqtari, M.A.A., Alghalibi, S.M., Alhamzy, E.H., 2011. Chemical composition and antimicrobial activity of essential oil of *Thymus vulgaris* from Yeme. Turk. J. Biochem. 36, 342–349.

Official Methods of Analysis of the Association of Official Analytical Chemist, eighteenth ed., 2012. AOAC, Washington, USA.

American Public Health Association (APHA), 2001. Compendium of Methods for the Microbiological Examination of Foods, fourth ed. (Washington, USA).

Asbahani, A.E., Miladi, K., Badri, W., Sala, M.A., Aït Addi, E.H., Casabianca, H., Mousadik, A.E., Hartmann, D., Jilale, A., Renaud, F.N.R., Elaissari, A., 2015. Essential oils: from extraction to encapsulation. Int. J. Pharm. 483, 220–243.

Asensio, C.M., Grosso, N.R., Juliani, H.R., 2015. Quality preservation of organic cottage cheese using oregano essential oils. LWT Food Sci. Technol. 60, 664–671.

Ballester-Costa, C., Sendra, E., Fernández-López, J., Pérez-Álvarez, J.A., Viuda-Martos, M., 2013. Chemical composition and *in vitro* antibacterial properties of essential oils of four *Thymus* species from organic growth. Ind. Crop. Prod. 50, 304–311.

Bouhdid, S., Abrini, J., Amensour, M., Zhiri, A., Espuny, M.J., Manresa, A., 2010. Functional and ultrastructural changes in *Pseudomonas aeruginosa* and *Staphylococcus aureus* cells induced by *Cinnamomum verum* essential oil. J. Appl. Microbiol. 109, 1139–1149.

Brazil, 1997. Ordinance No. 352 of 1997: Approves Technical Regulation for the Identity and Quality of "coalho" Cheese. Retrieved on 12 August 2014 from. http://mapa.gov.br.

Brazil, M.S., 2001. Technical Bulletin of Microbiological Analyses in Foods. Resolution RDC nº 12, January 2nd. Retrieved on 12 August 2014 from. http://www.anvisa.gov.br.

Brooks, J.C., Martinez, B., Stratton, J., Bianchini, A., Krokstrom, R., Hutkins, R., 2012. Survey of raw milk cheeses for microbiological quality and prevalence of foodborne pathogens. Food Microbiol. 31, 154–158.

Buriti, F.C.A., Okazaki, T.Y., Alegro, J.H.A., Saad, S.Y., 2007. Effect of a probiotic mixed

culture on texture profile and sensory performance of Minas fresh cheese in comparison with the traditional products. Arch. Lat. Amer. Nutr. 57, 179–185.
Burt, S.A., 2004. Essential oils: their antibacterial properties and potential applications in foods – a review. Int. J. Food Microbiol. 94, 223–253.
Carović-Stanko, K., Orlić, S., Politeo, O., Strikić, F., Kolak, I., Milos, M., Satovic, Z., 2010. Composition and antibacterial activities of essential oils of seven *Ocimum* taxa. Food Chem. 119, 196–201.
Clinical and Laboratory Standards Institute (CLSI), 2006. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. Approved Standard-Seventh Edition M07-A7. Wayne.
Coelho, M.C., Silva, C.C.G., Ribeiro, S.C., Dapkevicius, M.L.N.E., Rosa, H.J.D., 2014. Control of *Listeria monocytogenes* in fresh cheese using protective lactic acid bactéria. Int. J. Food Microbiol. 191, 53–59.
Di Pasqua, De Feo, V., Villane, F., Mauriello, G., 2005. In vitro antimicrobial activity of essential oils from mediterranean Apiaceae, Verbenaceae and Lamiaceae against foodborne pathogens and spoilage bacteria. Ann. Microbiol. 2, 139–143.
Food and Drug Administration, 2014. Antimicrobial Resistance: a Growing Threat, 2009. Retrieved on 15 December 2014 from. http://www.fda.gov.
Fox, P.F., Guinee, T.P., Cogan, T.M., Mcsweeney, P.L.H., 2000. Fundamentals of Cheese Science. Aspen Publishers, Gaithersburg.
Gandhi, M., Chikindas, M.L., 2007. Listeria: a foodborne pathogen that knows how to survive. Int. J. Food Microbiol. 113, 1–15.
Garcia, E.F., Oliveira, M.E.G., Queiroga, R.C.R.E., Machado, T.A.D., Souza, E.L., 2012. Development and quality of a Brazilian semi-hard goat cheese (coalho) with added probiotic lactic acid bacteria. Int. J. Food Sci. Nutr. 63, 947–956.
Gutierrez, J., Barry-Ryan, C., Bourke, P., 2008. The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. Int. J. Food Microbiol. 124, 91–97.
Gutierrez, J., Barry-Ryan, C., Bourke, P., 2009. Antimicrobial activity of plant essential oils using food model media: efficacy, synergistic potential and interactions with food components. Food Microbiol. 26, 142–150.
Herigstad, B., Hamilton, M., Heersink, J., 2011. How to optimize the drop plate method for enumerating bacteria. J. Microbiol. Methods 44, 121–129.
Janssen, A.M., Scheffer, J.J.C., Baerheim Svendsen, A.B., 1987. Antimicrobial activities of essential oils. A 1976-1986 literature review on possible applications. Pharm. Weekbl. (Sci.) 9, 193–197.
Kivanç, M., Akgül, A., Doğan, A., 1991. Inhibitory and stimulatory effects of cumin, oregano and their essential oils on growth and acid production of *Lactobacillus plantarum* and *Leuconostoc mesenteroides*. Int. J. Food Microbiol. 13, 81–85.
Kohiyama, C.Y., Ribeiro, M.M.Y., Mossini, S.A.G., Bando, E., Bomfim, N.S., Nerilo, S.B., Rocha, G.H.O., Grespan, R., Mikcha, J.M.G., Machinski, M., 2015. Antifungal properties and inhibitory effects upon aflatoxin production of *Thymus vulgaris* L. by *Aspergillus flavus* Link. Food Chem. 173, 1006–1010.
Laplante, K.L., 2007. In vitro activity of lysostaphin, mupirocin, and tea tree oil against clinical methicillin-resistant *Staphylococcus aureus*. Diagn. Microbiol. Infect. Dis. 57, 413–418.
López-Díaz, T.M., Alonso, C., Román, C., García-López, M.L., Moreno, B., 2000. Lactic acid isolates from a hand-made blue cheese. Food Microbiol. 17, 23–32.
McMahon, M.A.S., Tunney, M.M., Moore, J.E., Blair, I.S., Gilpin, D.F., 2008. Changes in antibiotic susceptibility in staphylococci habituated to sub-lethal concentrations of tea tree oil (*Melaleuca alternifolia*). Lett. Appl. Microbiol. 47, 263–268.
Mills, S., O'Suillvan, O., Hill, C., Fitzgerald, G., Ross, R.P., 2010. The changing face of dairy starter culture research: from genomics to economics. Int. J. Dairy Technol. 63, 149–170.
Monte, D.F.M., Tavares, A.G., Albuquerque, A.R., Sampaio, F.C., Oliveira, T.C.R.M., Franco, O.L., Souza, E.L., Magnani, M., 2014. Tolerance response of multidrug-resistant *Salmonella entérica* strains to habituation to *Origanum vulgare* L. essential oil. Front. Microbiol. 5, 1–6.
Nazer, A.I., Kobilinsky, A., Tholozan, J.L., Dubois-Brissonnet, F., 2005. Combination of food antimicrobials at low levels to inhibit the growth of *Salmonella* sv, typhimurium: a synergistic effect. Food Microbiol. 22, 391–398.
Neviani, E., De Dea Lindner, J., Bernini, V., Gatti, M., 2009. Recovery and differentiation of long ripened cheese microflora through a new cheese-based cultural medium. Food Microbiol. 26, 240–245.
Nezhadali, A., Nabavi, M., Rajabian, M., Akbarpour, M., Pourali, P., Amini, F., 2014. Chemical variation of leaf essential oil at different stages of plant growth and *in vitro* antibacterial activity of *Thymus vulgaris* Lamiaceae, from Iran. Beni-Suef Univ. J. Basic Appl. Sci. 3, 87–92.
Nowak, A., Kalemba, D., Krala, C., Piotrowska, M., Czyzowska, A., 2012. The effects of thyme (*Thymus vulgaris*) and rosemary (*Rosmarinus officinalis*) essential oils on *Brochothrix thermosphacta* and on the shelf life of beef packaged in high-oxygen modified atmosphere. Food Microbiol. 32, 212–216.
Oliveira, M.E.G., Garcia, E.F., Queiroga, R.C.R.E., Souza, E.L., 2012. Technological, physicochemical and sensory characteristics of a Brazilian semi-hard goat cheese (coalho) with added probiotic lactic acid bacteria. Sci. Agric. 69, 370–379.
Olmedo, R.H., Nepote, V., Grosso, N.R., 2013. Preservation of sensory and chemical properties in flavoured cheese prepared with cream cheese base using oregano and rosemary essential oils. LWT Food Sci. Technol. 53, 409–417.
Omidbeygi, M., Barzegar, M., Hamidi, Z., Naghdibadi, H., 2007. Antifungal activity of thyme, summer savory and cloves essential oil against *Aspergillus flavus* in liquid medium and tomato paste. Food Control 18, 1518–1523.
Pimentel-Filho, N.J., Mantovani, H.C., Carvalho, A.F., Dias, R.S., Vanetti, M.C.D., 2014. Efficacy of bovicin HC5 and nisin combination against *Listeria monocytogenes* and *Staphylococcus aureus* in fresh cheese. Int. J. Food Sci. Technol. 49, 416–422.
Queiroga, R.C.R.E., Santos, B.M., Gomes, A.M.P., Monteiro, M.J., Teixeira, S.M., Souza, E.L., Pereira, C.J.D., Pintado, M.M.E., 2013. Nutritional, textural and sensory properties of Coalho cheese made of goats', cows' milk and their mixture. LWT Food Sci. Technol. 50, 538–544.
Quero, G.M., Santovito, E., Visconti, A., Fusco, V., 2014. Quantitative detection of *Listeria monocytogenes* in raw milk and soft cheeses: culture-independent versus liquid and solid-based culture-dependent real time PCR approaches. LWT Food Sci. Technol. 28, 11–20.
Razzaghi-Abyaneh, M., Shams-Ghahfarokhi, M., Yoshinari, T., Rezaee, M.B., Jaimand, K., Nagasawa, H., Sakuda, S.H., 2008. Inhibitory effects of *Satureja hortensis* L. essential oil on growth and aflatoxin production by *Aspergillus parasiticus*. Int. J. Food Microbiol. 123, 228–233.
Sacchetti, G., Maietti, S., Muzzoli, M., Scaglianti, M., Manfredini, S., Radice, M., Bruni, R., 2005. Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods. Food Chem. 91, 621–632.
Sant'Ana, A.M.S., Bezerril, F.F., Madruga, M.S., Batista, A.S.M., Magnani, M., Sousa, E.L., Queiroga, R.C.R.E., 2013. Nutritional and sensory characteristics of Minas fresh cheese made with goat milk, cow milk, or a mixture of both. J. Dairy Sci. 96, 7442–7453.
Shelef, L.A., 1983. Antimicrobial effect of spices. J. Food Saf. 6, 29–44.
Shelef, L.A., Naglik, O.A., Bogen, D.W., 1980. Sensitivity of some common food-borne bactéria to the spices sage, rosemary, and allspice. J. Food Sci. 45, 1042–1044.
Smith, E.C.J., Williamson, E.M., Wareham, N., Kaatz, G.W., Gibbons, S., 2007. Antibacterials and modulators of bacterial resistance from the immature cones of *Chamaecyparis lawsoniana*. Phytochemistry 68, 210–217.
Sousa, J.P., Torres, R.A., Azerêdo, G.A., Figueiredo, R.C.B.Q., Vasconcelos, M.A.S., Souza, E.L., 2012. Carvacrol and 1,8-cineole alone or in combination at sublethal concentrations induce changes in the cell morphology and membrane permeability of *Pseudomonas fluorescens* in a vegetable-based broth. Int. J. Food Microbiol. 158, 9–13.
Teuber, M., 1995. The genus lactococcus. In: Wood, B.J.B., Holzapfel, W.H. (Eds.), The Genera of Lactic Acid Bacteria, vol. 2. Chapman & Hall, London, pp. 235–278.
Tiwari, K.P., Pandey, A., 1981. Effect of some spices on acid production by starter cultures. J. Food Prot. 42, 572–576.
Tohidpour, A., Sattari, M., Omidbaigi, R., Yadegar, A., Nazemi, J., 2010. Antibacterial effect of essential oils from two medicinal plants against methicillin – resistant *Staphylococcus aureus* (MRSA). Phytomedicine 17, 142–145.
Van Vuuren, S.F., 2008. Antimicrobial activity of South African medicinal plants. J. Ethnopharmacol. 119, 462–472.
Wiegand, I., Hilpert, K., Hancock, R.E.W., 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat. Protoc. 3, 163–175.
Zeleny, R., Emteborg, H., Charoud-Got, J., Schimmel, H., Nia, Y., Mutel, I., Ostyn, A., Herbin, S., Hennekinne, J.A., 2015. Development of a reference material for *Staphylococcus aureus* enterotoxin A in cheese: feasibility study, processing, homogeneity and stability assessment. Food Chem. 168, 241–246.